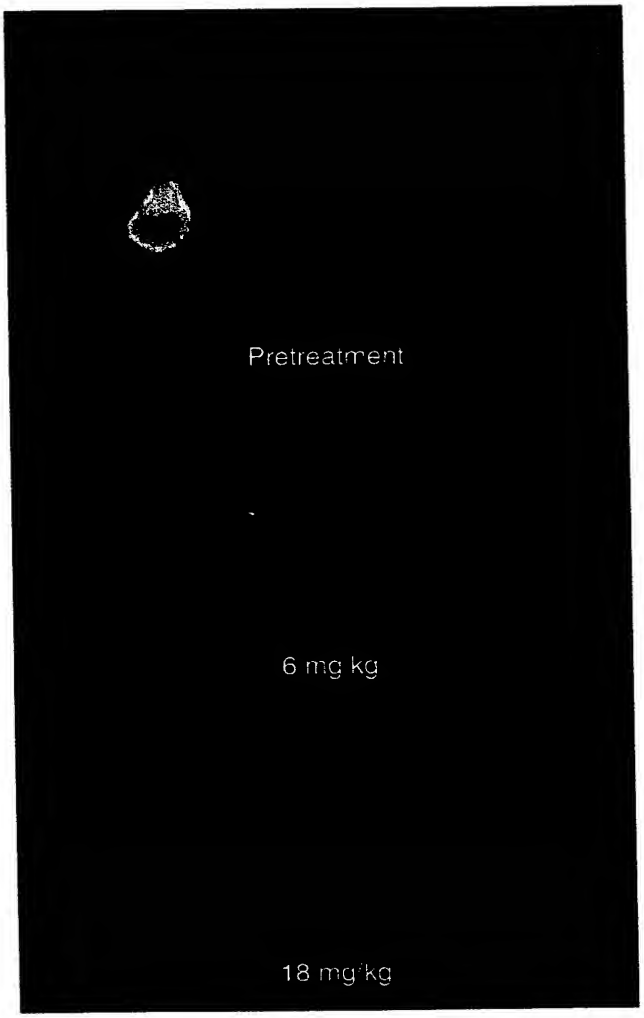
Cancer Research



PET Imaging of αvβ3 Integrin Expression

March 1, 2001
Volume 61 • Number 5
www.aacr.org

AACR American Association for Cancer Research

[CANCER RESEARCH 61, 2424–2428, March 15, 2001]

Advances in Brief

Ligands for Peroxisome Proliferator-activated Receptors α and γ Inhibit Chemically Induced Colitis and Formation of Aberrant Crypt Foci in Rats[1]

Takuji Tanaka,[2,3] Hiroyuki Kohno,[2] Shin-ichiro Yoshitani, Shigeki Takashima, Ataru Okumura, Akira Murakami, and Masashi Hosokawa

Departments of Pathology [T. T., H. K.] and General and Gastrointestinal Surgery [S-i. Y., S. T.], Kanazawa Medical University, Ishikawa 920-0293; Department Endocrinological Metabolism, Anjo Kosei Hospital, Aichi 446-8602 [A. O.]; Department of Biotechnological Science, Faculty of Biology-Oriented Science and Technology, K University, Wakayama 649-6493 [A. M.]; and Graduate School of Fisheries Sciences, Hokkaido University, Hokkaido 041-8611 [M. H.], Japan

Abstract

The biological role of the peroxisome proliferator-activated receptors (PPARs) in various diseases, including inflammation and cancer, has been highlighted recently. Although PPARγ ligands have been found to inhibit mammary carcinogenesis in rodents, the effects on colon tumorigenesis are controversial. In the present study, three different experiments were conducted to investigate the modifying effects of PPARs ligands (PPARα and PPARγ) on colitis and an early phase of colitis-related colon carcinogenesis in male F344 rats. In the first experiment, gastric gavage of troglitazone (PPARγ ligand, 10 or 100 mg/kg body weight) or bezafibrate (PPARα ligand, 10 or 100 mg/kg body weight) inhibited colitis induced by dextran sodium sulfate (DSS) and lowered trefoil factor-2 content in colonic mucosa. In the second experiment, dietary administration (0.01 or 0.05% in diet) of troglitazone and bezafibrate for 4 weeks significantly reduced azoxymethane (AOM, two weekly s.c. injections, 20 mg/kg body weight)-induced formation of aberrant crypts foci, which are precursor lesions for colon carcinoma. In the third experiment, dietary administration (0.01% in diet for 6 weeks) of pioglitazone (PPARγ ligand), troglitazone, and bezafibrate effectively suppressed DSS/AOM-induced ACF. Administration of both ligands significantly reduced cell proliferation activity in colonic mucosa exposed to DSS and AOM. Our results suggest that synthetic PPARs ligands (PPARα and PPARγ) can inhibit the early stages of colon tumorigenesis with or without colitis.

Introduction

Patients with long-standing ulcerative colitis are at increased risk of developing colorectal cancer compared with the general population (1). Thus, prevention of colonic malignancy by inhibition of ulcerative colitis is an attractive approach in the overall management of colonic cancer (2, 3).

Acute and chronic inflammatory processes may be important among the factors involved in oxidative events leading to DNA damage in the colorectal epithelium (4). DSS[4] can be used to induce both acute and chronic colonic inflammations, depending on the d and duration of treatment (5). Long-term administration of D induces colorectal cancer (6), whereas short-term exposure result the formation of ACF (7), which are precursor lesions for co adenocarcinoma (8–10), and enhances the development colore cancer. Therefore, the DSS-induced colitis model is useful for stu ing colitis-associated colorectal neoplasia (3, 11).

Recently, the physiological function and metabolism of PPA which are members of the nuclear hormone receptor superfamily well as their role in various nutritional states and various dise including inflammation and cancer have been highlighted (revie in Refs. 12–15). PPARs bind to a specific element in the prom region of target genes. PPAR and other nuclear hormone recep bind the promoter only as a heterodimer with the receptor for 9- retinoic acid, retinoid X receptor. They activate transcription in sponse to binding of the hormone (ligand). Eicosanoids and fatty a can regulate gene transcription through PPARs. At present, sev PPARs have been identified including PPARα, PPARβ (PPA NUC-1, or FAAR), and PPARγ. Some ligands, such as polyuns rated fatty acids and probably oxidized fatty acids, are shared by three isotypes. Several compounds including linoleic acid, phyt acid, conjugated linolenic acid, 8*S*-hydroxyeicosatetraenoic acid, leukotriene B_4 bind with high affinity to PPARα. PPARs are pressed in the intestine at various levels (16, 17). The ligands PPARγ are suspected to modify carcinogenesis (15). In colon c nogenesis, conflicting results between *in vivo* (18, 19) and *in* (20) studies have been reported on the action of synthetic PP agonists, troglitazone and rosiglitazone, used for the treatment of II diabetes (21). *In vivo* studies of other organs showed that GW7 a new PPARγ ligand, could inhibit rat mammary carcinogenesis (DuBois *et al.* (23) reported aberrant expression of PPARγ in ch ically induced colonic carcinoma and human colon cancer cell li Similar to PPARα (24), PPARγ agonists 15-deoxy-$\Delta^{12,14}$-pros landin J_2 and troglitazone exhibit anti-inflammatory properties 26). Moreover, PPARγ ligand effectively inhibits colitis in mice (Interestingly, NSAIDs including indomethacin, which are candi chemopreventive agents against colon cancer, activate both PP and PPARγ (28).

Suppression of tumor growth *in vitro* (20) is not supporte animal models of familial adenomatous polyposis coli (18, 19). I present study, we investigated the role of PPARα and PPARγ lig in preventing colitis induced by DSS and ACF induced by DSS ar AOM in rats. Because TFF play an important role in the repair healing of the gastrointestinal tract (29), we measured TFF2 and T concentrations in the mucosa of rats with experimentally ind colitis. Because control of cell proliferation is important for ca inhibition (30), we also estimated the cell proliferation activi

Received 11/8/00; accepted 1/31/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grants-in-Aid for Scientific Research 12213131 and 10671782 from the Ministry of Education, Culture, Sports, and Technology of Japan; by a Grant-in Aid for the Second Term for a Comprehensive 10-year Strategy for Cancer Control from the Ministry of Health and Welfare, Japan; Grant HS-52260 for the Comprehensive Research Project on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation; and Grant H2000-4 for Project Research from High-Technology Center of Kanazawa Medical University.

[2] These two authors contributed equally to this work.

[3] To whom requests for reprints should be addressed, at First Department of Pathology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan. E-mail: takutt@kanazawa-med.ac.jp.

[4] The abbreviations used are: DSS, dextran sodium sulfate; ACF, aberrant crypt foci; PPAR, peroxisome proliferator-activated receptor; NSAID, nonsteroidal anti-inflammatory drug; AOM, azoxymethane; TFF, trefoil factor; AgNOR, silver-stained nucleolar organizer region; COX, cyclooxygenase.

colonic mucosa exposed to DSS, AOM, and PPARs ligands by counting the number of AgNOR protein per mucosal cell nucleus (AgNORs index). Our results showed that PPARα and PPARγ ligands suppressed DSS-induced colitis and DSS- and/or AOM-induced ACF. This may explain the protective role of ligands for PPARα and PPARγ against colonic ACF and suggests a possible therapeutic effect in colitis-associated colon carcinogenesis.

Materials and Methods

Animals, Chemicals, and Diets. Male F344 rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan), 4 weeks of age, were used in three different experiments. The animals were maintained at Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages (three or four rats/cage) with free access to drinking water and a basal diet, CE-2 (CLEA Japan Inc., Tokyo, Japan), under controlled conditions of humidity (50 ± 10%), light (12/12 h light/dark cycle), and temperature (23 ± 2°C). They were quarantined for the first 14 days and then randomized by body weight into experimental and control groups. DSS, with a molecular weight of 40,000, was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). DSS for induction of colitis was dissolved in water at a concentration of 1% (w/v). AOM for induction of ACF was purchased from Sigma Chemical Co. (St. Louis, MO). Powdered CE-2 diet was used as a basal diet throughout the study. PPARs ligands tested included PPARγ ligand troglitazone (Sankyo Co., Tokyo), PPARγ ligand pioglitazone (Takeda Chemical Industries, Osaka, Japan), and PPARα ligand bezafibrate (Kissei Pharmaceutical Co., Matsumoto, Japan). Experimental diets containing troglitazone, pioglitazone, and bezafibrate were prepared by mixing the respective compound in powdered basal diet CE-2 at a concentration (w/w) of 0.01 or 0.05%.

Experimental Procedure. Three experiments were conducted to examine the effects of PPAR ligands in rats with DSS-induced colitis, AOM-induced ACF, and DSS/AOM-induced ACF.

Experiment 1. A total of 54 male F344 rats were divided into eight experimental and control groups (Fig. 1*A*). Groups 1–5 were provided with drinking water containing 1% DSS *ad libitum* for 7 days. Groups 2–5 also received gastric gavage of troglitazone (10 mg/kg body weight for group 2 or 100 mg/kg body weight for group 3) or bezafibrate (10 mg/kg body weight for group 4 or 100 mg/kg body weight for group 5) suspended in 0.75% methyl cellulose containing normal physiological solution every other day for 3 times, starting 24 h after commencement of the study. Groups 6 and 7 were treated with troglitazone and bezafibrate (100 mg/kg body weight), respectively. Group 8 represents untreated control rats. The experiment was terminated at day 8. All animals were sacrificed, and the colons were flushed with saline, excised, cut open longitudinally along the main axis, and then washed with saline. The colon was cut and fixed in 10% buffered formalin for at least 24 h. Histological examination was performed using paraffin-embedded sections after H&E staining. For statistical evaluation, the severity of colonic inflammation was histologically scored in a blind fashion according to the scoring system (31): grade 0, normal; grade 1, focal inflammatory cell infiltration including neutrophils; grade 2, crypt loss with inflammatory cell infiltration or crypt abscess formation; grade 3, mucosal ulceration, or five or more foci of gland loss with inflammatory cell infiltration; and grade 4, two or more areas of mucosal ulceration. At the end of the study, the concentrations of TFF2 and TFF3 in the colonic mucosa of three rats from each group were measured by RIA (32) using region-specific polyclonal antibodies in each case. Total protein concentration was also determined using the protocol of Bradford (33).

Experiment 2. A total of 56 male F344 rats were divided into eight experimental and control groups (Fig. 1*B*). Groups 1–5 were treated with AOM by two weekly s.c. injections (20 mg/kg body weight). Rats in groups 2 and 3 were fed a diet containing 0.01 and 0.05% troglitazone, respectively, for 4 weeks, commencing 1 week before the first dose of AOM. Groups 4 and 5 were fed a diet mixed with 0.01 and 0.05% bezafibrate, respectively, for 4 weeks, commencing 1 week before the first injection of AOM. Groups 6 and 7 were given a diet containing 0.05% troglitazone and bezafibrate alone. Group 8 served as the untreated control. Rats were sacrificed at week 4 by ether overdose to assess the incidence of colonic ACF. The frequency of ACF was determined according to the method described in our previous report (10). At necropsy, the colons were flushed with saline, excised, cut open longitudinally along the main axis, and then washed with saline. They were cut and fixed in

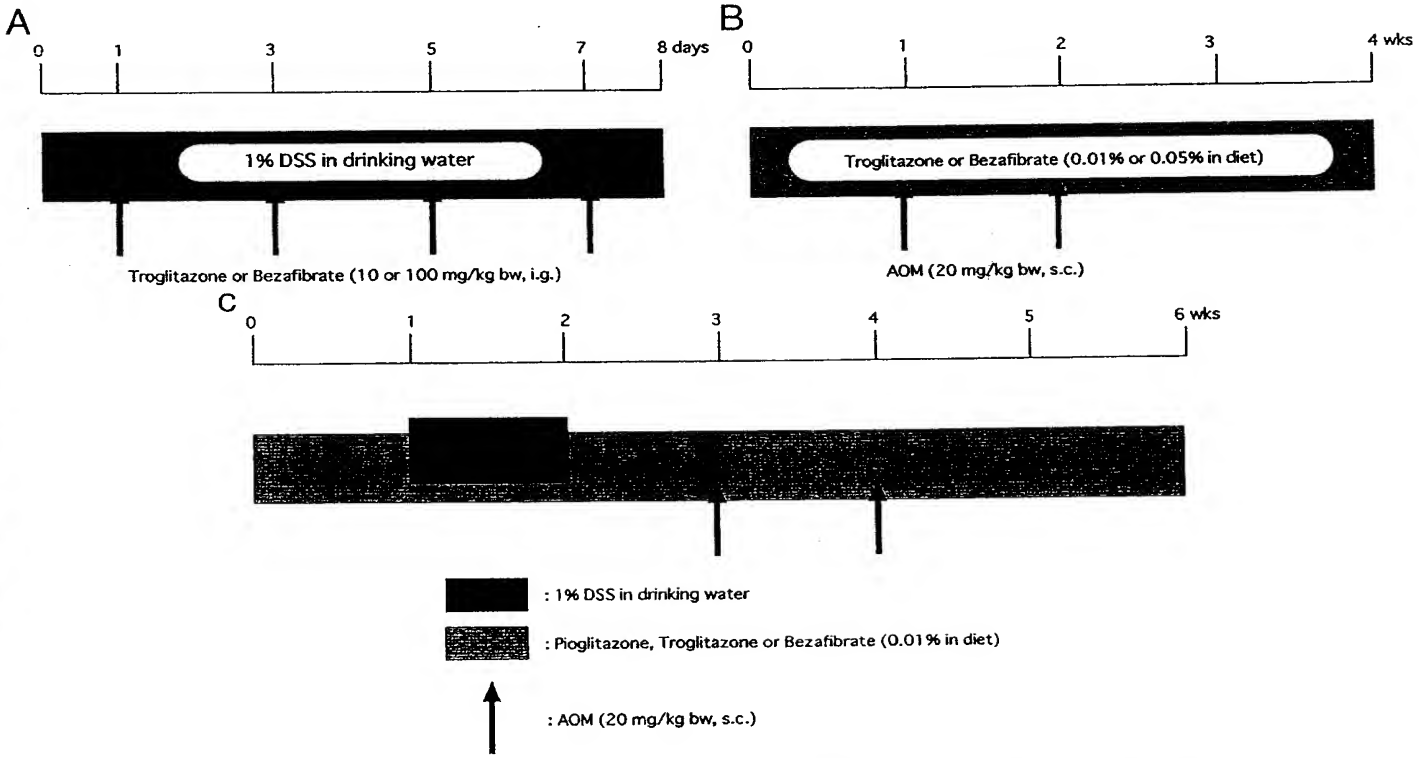


Fig. 1. Experimental protocols. *A*, experiment 1; *B*, experiment 2; *C*, experiment 3. *bw*, body weight; *i.g.*, intragastrically.

Table 1 *Effects of PPARs ligands on DSS-induced colitis (experiment 1)*

Group no.	Treatment	*n*	Body weight(g)	Liver weight (g)	Relative liver weight[a]	Score of colitis
1	1% DSS	10	154 ± 6[b]	8.4 ± 1.1	5.45 ± 0.58	3.60 ± 0.66
2	1% DSS/troglitazone (10 mg/kg bw i.g.)	8	148 ± 10	8.5 ± 1.5	5.73 ± 0.90	1.88 ± 0.78[c]
3	1% DSS/troglitazone (100 mg/kg bw i.g.)	8	149 ± 9	8.5 ± 1.2	5.75 ± 0.98	1.00 ± 0.87[d]
4	1% DSS/bezafibrate (10 mg/kg bw i.g.)	8	148 ± 11	8.3 ± 0.8	5.67 ± 0.67	1.88 ± 1.05[e]
5	1% DSS/bezafibrate (100 mg/kg bw i.g.)	8	147 ± 3	9.1 ± 0.8	6.22 ± 0.54	1.50 ± 1.00[f]
6	Troglitazone (100 mg/kg bw i.g.)	4	158 ± 3	8.2 ± 0.9	5.23 ± 0.67	0
7	Bezafibrate (100 mg/kg bw i.g.)	4	153 ± 7	9.6 ± 1.8	6.29 ± 0.93	0
8	None	4	159 ± 5	8.3 ± 1.1	5.22 ± 0.59	0

[a] Liver weight/100 g body weight. bw, body weight; i.g., intragastrically.
[b] Mean ± SD.
[c–f] *Significantly different from group 1:* [c] $P < 0.005$; [d] $P < 0.001$; [e] $P < 0.02$; and [f] $P < 0.002$.

10% buffered formalin for at least 24 h. Fixed colons were dipped in 0.5% solution of methylene blue in distilled water for 30 s, briefly washed with distilled water, and placed on a microscope slide with the mucosal surface up. Using a light microscope at a magnification of ×40, ACF were distinguished from the surrounding "normal-appearing" crypts by their large size (10).

Experiment 3. A total of 55 male F 344 rats were divided into nine experimental and control groups (Fig. 1*C*). Group 1 was administered two weekly s.c. injections of AOM on weeks 3 and 4 at a dose of 20 mg/kg body weight. Groups 2–5 were administered 1% DSS in drinking water for 7 days and two weekly s.c. injections of AOM (20 mg/kg body weight), commencing 7 days after DSS administration. Groups 3, 4, and 5 were fed diets containing 0.01% pioglitazone, troglitazone, and bezafibrate for 6 weeks, respectively. Groups 6–8 were administered diets mixed with 0.01% pioglitazone, troglitazone, and bezafibrate alone, respectively. Group 9 consisted of untreated control rats. The severity of colitis and development of ACF were evaluated at week 6, as described in Experiments 1 and 2. In addition, the AgNORs index was counted on one-step silver-stained sections (34) in four rats in each of groups 1–5 and in three rats in each of groups 6–9. Measurements were performed on 25 crypts/rat.

Statistical Analysis. All data were expressed as mean ± SD. Data were compared by one-way ANOVA, followed by a Bonferroni/Dunn post-hoc test. $P < 0.05$ denoted the presence of a statistically significant difference.

Results

Experiment 1. The mean body weights of rats of groups 2–5, which were treated with 1% DSS and troglitazone or bezafibrate, were slightly lower than that of group 1 treated with 1% DSS alone at th end of the study (Table 1). The mean liver and relative liver weight did not differ among the groups. Scoring of severity of colitis showe significant reduction in groups 2–5, compared with group ($P < 0.005$, $P < 0.001$, $P < 0.02$, and $P < 0.002$, respectively). Th concentrations of TFF2 and TFF3 in colonic mucosa are shown i Table 2. Although the concentrations of TFF3 were comparabl among the groups, those of TFF2 in groups 2–5 were lower than tha of group 1, and the value of group 3 was significantly smaller tha group 1 ($P < 0.05$).

Experiment 2. Body, liver, and relative liver weights at the end o the experiment are shown in Table 3. The mean body weight of grou 1 (AOM alone) was significantly lower ($P < 0.001$) than group (untreated). Mean liver and relative liver weights of rats amon groups 1–6 did not significantly differ. Dietary administration o 0.05% bezafibrate significantly elevated the mean liver and relativ liver weights compared with the untreated control ($P < 0.05$ an $P < 0.01$, respectively).

ACF analysis showed that administration of 0.05% troglitazone o bezafibrate caused a significant reduction in the numbers of ACF colon ($P < 0.01$ or $P < 0.001$), as shown in Table 3. Administratio of 0.05% troglitazone significantly reduced aberrant crypts/focu ($P < 0.005$).

Table 2 *Colonic tissue concentrations of endogenous TFF2 and TFF3 (experiment 1)*

Group no.	Treatment	*n*	TFF2 (pmol/mg protein)	TFF3 (pmol/mg protein)
1	1% DSS	3	2.13 ± 0.24[a,b]	105.0 ± 33.3
2	1% DSS/troglitazone (10 mg/kg bw i.g.)	3	1.60 ± 0.14	102.7 ± 35.6
3	1% DSS/troglitazone (100 mg/kg bw i.g.)	3	0.92 ± 0.15[c]	94.0 ± 31.1
4	1% DSS/bezafibrate (10 mg/kg bw i.g.)	3	1.66 ± 0.16	100.3 ± 31.3
5	1% DSS/bezafibrate (100 mg/kg bw i.g.)	3	1.01 ± 0.18	97.9 ± 23.7
6	Troglitazone (100 mg/kg bw i.g.)	3	0.68 ± 0.08	59.3 ± 13.3
7	Bezafibrate (100 mg/kg bw i.g.)	3	0.71 ± 0.07	58.4 ± 6.7
8	None	3	0.67 ± 0.07	56.0 ± 8.9

[a] Mean ± SD. bw, body weight; i.g., intragastrically.
[b] Significantly different from group 8 ($P < 0.02$).
[c] Significantly different from group 1 ($P < 0.05$).

Table 3 *Effects of PPAR ligands on AOM-induced ACF formation in male F344 rats (experiment 2)*

Group no.	Treatment (no. of rats examined)	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)	ACF/colon	ACs/focus
1	AOM alone (12)	166 ± 3[a,b]	10.3 ± 1.4	4.73 ± 0.49	83 ± 6	2.01 ± 0.24
2	AOM + 0.01% troglitazone (8)	172 ± 11	10.8 ± 1.0	4.89 ± 0.44	68 ± 16	1.66 ± 0.21
3	AOM + 0.05% troglitazone (8)	176 ± 5[c]	11.1 ± 1.0	4.99 ± 0.28	55 ± 13[d]	1.54 ± 0.13[e]
4	AOM + 0.01% bezafibrate (8)	181 ± 5[c]	12.3 ± 1.1	5.38 ± 0.23	75 ± 8	1.97 ± 0.20
5	AOM + 0.05% bezafibrate (8)	178 ± 9	11.8 ± 0.5	4.88 ± 0.19	53 ± 9[c]	1.85 ± 0.10
6	0.05% troglitazone (4)	194 ± 3	9.2 ± 0.8	4.72 ± 0.36	0	0
7	0.05% bezafibrate (4)	193 ± 9	12.3 ± 0.5[f]	6.34 ± 0.14[g]	0	0
8	None (4)	182 ± 6	9.0 ± 1.0	4.89 ± 0.33	0	0

[a] Mean ± SD.
[b,f,g] Significantly different from group 8: [b] $P < 0.001$; [f] $P < 0.05$; and [g] $P < 0.01$.
[c–e] Significantly different from group 1: [c] $P < 0.001$; [d] $P < 0.01$; and [e] $P < 0.005$.

Table 4 *Effects of PPAR ligands on AOM and/or DSS-induced ACF formation in male F344 rats (experiment 3)*

Group no.	Treatment (*n*)	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)	ACF/colon	ACs/focus	AgNORs index
1	AOM alone (8)	234 ± 7[a]	10.5 ± 1.4	4.5 ± 0.49	84 ± 13	2.0 ± 0.21	1.6 ± 0.21[b]
2	1% DSS + AOM (10)	226 ± 7	11.0 ± 1.5	4.9 ± 0.65	115 ± 22	2.4 ± 0.29	1.9 ± 0.13
3	1% DSS + AOM + 0.01% pioglitazone (7)	231 ± 17	11.0 ± 0.6	4.8 ± 0.34	71 ± 24[c]	1.8 ± 0.17[d]	1.2 ± 0.22[e]
4	1% DSS + AOM + 0.01% troglitazone (7)	219 ± 8	9.7 ± 0.6	4.4 ± 0.33	57 ± 14[e]	1.6 ± 0.14[e]	0.9 ± 0.15[e]
5	1% DSS + AOM + 0.01% bezafibrate (7)	216 ± 9	14.2 ± 1.8[c]	6.5 ± 0.63[d]	59 ± 18[f]	1.7 ± 0.16[g]	0.9 ± 0.16[e]
6	0.01% pioglitazone (4)	250 ± 7	9.7 ± 1.4	3.9 ± 0.57	0	0	0.8 ± 0.12
7	0.01% troglitazone (4)	222 ± 8	11.0 ± 1.0	4.9 ± 0.36	0	0	0.7 ± 0.19
8	0.01% bezafibrate (4)	225 ± 6	11.7 ± 0.6	5.2 ± 0.26	0	0	0.7 ± 0.11
9	None (4)	221 ± 8	10.2 ± 1.0	4.5 ± 0.34	0	0	0.7 ± 0.09

[a] Mean ± SD.
[b] Significantly different from group 9: $P < 0.001$.
[c–g] $^{c}P < 0.05$; $^{d}P < 0.01$; $^{e}P < 0.001$; $^{f}P < 0.005$; and $^{g}P < 0.002$.

Experiment 3. As shown in Table 4, the final mean body weight of group 1 (AOM alone) was slightly higher than that of group 9 (untreated). The mean body weights of all groups did not significantly differ. Administration of 0.01% bezafibrate (group 5) significantly increased liver ($P < 0.05$) and relative liver weights ($P < 0.01$).

ACF analysis (Table 4) showed that treatment with 1% DSS and AOM (group 2) elevated the number of ACF/colon and aberrant crypts/focus. In groups 3–5, which were treated with 1% DSS, AOM, and 0.01% pioglitazone (group 3), 0.01% troglitazone (group 4), or 0.01% bezafibrate (group 5), both biomarkers were significantly smaller than those of group 2 ($P < 0.05$, $P < 0.001$, $P < 0.005$, $P < 0.01$, or $P < 0.002$). As summarized in Table 4, the mean AgNORs index of group 1 (AOM alone) was significantly higher than group 9 (untreated; $P < 0.001$). DSS administration increased this value. Treatment with PPAR ligands (groups 3–5) significantly decreased AgNORs index compared with group 2 ($P < 0.001$).

Discussion

The results of the present study clearly indicated that PPAR ligands (either PPARγ or PPARα) inhibited DDS-induced colitis and AOM-induced ACF. In addition, both ligands inhibited DSS/AOM-induced ACF formation, which are precursor lesions for colon carcinoma in the presence of colitis (7). Our data on inhibition of chemically induced colitis are in agreement with those reported by Su *et al.* (27). The results of experiments on ACF formation support those of Sarraf *et al.* (20), in which troglitazone reduced the growth rate and induced differentiation of human colon tumor cell lines, both in culture and in nude mice. To our knowledge, this is the first report demonstrating that PPARs ligands (PPARγ or PPARα) could inhibit colitis and ACF formation.

In Experiment 1, both troglitazone (a PPARγ ligand) and bezafibrate (a PPARα ligand) effectively inhibited DSS-induced colitis. TFF2 and TFF3 are known to protect gastrointestinal injury (29). In the large intestine with normal or pathological conditions, TFF3 but not TFF2 is expressed (29). However, in the present study, PPAR ligands decreased the concentration of TFF2 but not TFF3 during the healing stage of colitis induced by DSS. Similar findings were reported in another model of colitis (35). In Experiments 2 and 3, administration of bezafibrate significantly inhibited AOM-induced and DSS/AOM-induced colonic ACF. The treatment caused hepatomegaly, but no preneoplastic or neoplastic lesions were identified in the liver. In Experiment 3, the frequency of ACF in rats treated with DSS and AOM was greater than that of AOM alone, suggesting that colitis caused by DSS enhanced ACF formation initiated by AOM. Such enhancing effects of inflammation on colon carcinogenesis has also been reported by other researchers (31).

Given the correlation between increased COX-2 expression and colonic carcinoma and/or inflammation, the chemopreventive effects of NSAIDs seems to be mediated, at least in part, by COX inhibition (36). We demonstrated previously that indomethacin, a NSAID, inhibited colon tumorigenesis as well as colitis induced by a naturally occurring carcinogen, 1-hydroxyanthraquinone (37). Interestingly, some NSAIDs act as peroxisome proliferators (28), suggesting that they might also regulate gene expression as part of their chemopreventive mechanism. Inhibition of colonic inflammation and decrease in cell proliferation by PPARs ligands might be responsible for their chemopreventive effects on colitis-associated colon carcinogenesis, as seen in Experiment 3. Damage to DNA by reactive oxygen and nitrogen species contributes to inflammatory diseases, including colitis-related colon tumorigenesis (4). PPARα (24) and PPARγ (25) are involved in inflammation control, and both inhibit inducible nitric oxide synthase (26, 38). Several NSAIDs bind to PPARα and PPARγ (28). Their anti-inflammatory activities might be mediated through inhibition of COX-1 and/or COX-2. PPARα could depress COX-2 induction (15). Therefore, activation of this PPAR may contribute to the anti-inflammatory activity of these drugs. Furthermore, immunomodulation by the ligands (12) might contribute to inhibition of colitis and colon carcinogenesis.

PPARγ is aberrantly expressed in chemically induced rodent colon cancer and in several human colon cancer cell lines (23). It can be up-regulated by treatment with butyrate, which induces differentiation of colon cancer cell line, Caco-2 cells (39). Development of human colon cancer is often associated with mutations in *PPARγ* gene (40). In addition, Lehmann *et al.* (28) reported that NSAIDs could bind and activate PPARγ, providing the molecular basis for the preventive effects of these drugs in colon carcinogenesis. In their study, NSAIDs also activated PPARα. The results of the present study (experiments 2 and 3), in which ligands for PPARα and PPARγ effectively prevented the development of ACF, lend support to the above findings. Although PPARγ is the predominant type of PPARs in the colon (16, 17) and its expression increases upon differentiation in human colonic adenocarcinoma cells (41), PPARα is also expressed in the colon (16) and could differentiate malignant tumor cells (42).

Recently, Suh *et al.* (22) reported that a new ligand for PPARγ (GW7845) inhibits nitrosomethylurea-induced mammary carcinogenesis in female rats. Subsequently, Mehta *et al.* (43) reported that troglitazone effectively inhibited preneoplastic mammary lesions in female BALB/c mice induced by 7,12-dimethylbenz[*a*]anthracene. Importantly, the inhibitory effects noted in their study were enhanced by treatment with a retinoid X receptor-selective retinoid, LG10068 (43). Although the effects of PPARγ ligands on intestinal tumorigenesis are conflicting (18–20), the results of the present study (experiments 2 and 3) suggest possible chemopreventive effects by PPARα and PPARγ ligands in colon tumorigenesis with or without colitis.

Because troglitazone can cause hepatic dysfunction (44), other PPARγ ligands, such as pioglitazone and GW7845, deserve further investigation with respective to their colon cancer chemopreventive effects. Interesting reports have been described recently. Human colon cancer cells express low levels of 13-*S*-hydroxyoctadecadienoic acid, a potent natural ligand of PPARγ, and 15-lipoxygenase-1, suggesting that 13-*S*-hydroxyoctadecadienoic acid production via 15-lipoxygen-

ase-1 down-regulation plays a role in colon tumorigenesis (45). As for PPARα ligands, some (fibrates, phthalate ester plasticisers, pesticides, and hypolipidemic drugs) are considered to be nongenotoxic hepatic carcinogens in rodents (46), although these data are not relevant to humans (47). Thus, PPARα ligands are also candidate chemopreventive agents against colon carcinogenesis.

In conclusion, we demonstrated in the present study that synthetic PPARs ligands (PPARα and PPARγ) could inhibit DSS-induced colitis, AOM-induced ACF, and DSS/AOM-induced ACF. PPARγ ligands could induce growth arrest and differentiation in human colon cancer cell lines (41). In addition, these ligands may inhibit tumor angiogenesis (48). Combined together, the present results and those of previous studies suggest that PPAR activation may be beneficial through inhibition of the early and/or late stages of colon tumorigenesis.

Acknowledgments

We thank Ayumi Nagaoka for secretarial assistance. We also express our thanks to the staff of the Research Animal Facility.

References

1. Itzkowitz, S. H. Colorectal cancer in inflammatory bowel disease. *In:* W. Schmiegel and J. Scholmerich (eds.), Colorectal Cancer: Molecular Mechanisms, Premalignant State and Its Prevention, pp. 118–126. Dordrecht, the Netherlands: Kluwer Academic Publishers BV, 1999.
2. Kakizoe, T. Asian studies of cancer chemoprevention: latest clinical results. Eur. J. Cancer, *36:* 1303–1309, 2000.
3. Tanaka, T., Kohno, H., Murakami, M., Shimada, R., and Kagami, S. Colitis-related rat colon carcinogenesis induced by 1-hydroxyanthraquinone and methylazoxymethanol acetate. Oncol. Rep., *7:* 501–508, 2000.
4. Maeda, H., and Akaike, T. Review: nitric oxide and oxygen radicals in infection, inflammation, and cancer. Biochemistry (Moscow), *63:* 854–865, 1998.
5. Iwai, A., and Iwasgita, E. Changes in colonic inflammation induced by dextran sulfate sodium (DSS) during short- and long-term administration of rebamipide. Dig. Dis. Sci., *43:* 143S–147S, 1998.
6. Hirono, I., Kuhara, K., Yamaji, T., Hosaka, S., and Golberg, L. Induction of colorectal squamous cell carcinomas in rats by dextran sulfate sodium. Carcinogenesis (Lond.), *3:* 353–355, 1992.
7. Whiteley, L. O., Hudson, L. J., and Pretlow, T. P. Aberrant crypt foci in the colonic mucosa of rats treated with a genotoxic and nongenotoxic colon carcinogen. Toxicol. Pathol., *24:* 681–689, 1996.
8. Pretlow, T. P., Barrow, B. J., Ashton, W. S., O'Riordan, M. A., Pretlow, T. G., Jurcisek, J. A., and Stellato, T. A. Aberrant crypts: putative preneoplastic foci in human colonic mucosa. Cancer Res., *51:* 1564–1567, 1991.
9. Pretlow, T. P., O'Riordan, M. A., Somich, G. A., Amini, S. B., and Pretlow, T. G. Aberrant crypts correlate with tumor incidence in F344 rats treated with azoxymethane and phytate. Carcinogenesis (Lond.), *13:* 1509–1512, 1992.
10. Bird, R. P. Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. Cancer Lett., *93:* 55–71, 1995.
11. Cooper, H. S., Murthy, S., Kido, K., Yoshitake, H., and Flanigan, A. Dysplasia and cancer in the dextran sulfate sodium mouse colitis model. Relevance to colitis-associated neoplasia in the human: a study of histopathology, β-catenin and p53 expression and the role of inflammation. Carcinogenesis (Lond.), *21:* 757–768, 2000.
12. Gelman, L., Fruchart, J-C., and Auwerx, J. An update on the mechanisms of action of the peroxisome proliferator-activated receptors (PPARs) and their roles in inflammation and cancer. Cell. Mol. Life Sci., *55:* 932–943, 1999.
13. Auwerx, J. *PPARγ*, the ultimate thrifty gene. Diabetologia, *42:* 1033–1049, 1999.
14. Kersten, S., Desvergne, B., and Wahli, W. Roles of PPARs in health and disease. Nature (Lond.), *405:* 421–424, 2000.
15. Vamecq, J., and Latruffe, N. Medical significance of peroxisome proliferator-activated receptor. Lancet, *354:* 141–148, 1999.
16. Mansen, A., Guardiola-Diaz, H., Rafter, J. C. B., and Gustafsson, J-A. Expression of the peroxisome proliferator-activated receptor (PPAR) in the mouse colonic mucosa. Biochem. Biophys. Res. Commun., *222:* 844–851, 1996.
17. Jain, S., Pulikuri, S., Zhu, Y., Qi, C., Kanwar, Y. S., Yeldandi, A. V., Rao, M. S., and Reddy, J. K. Differential expression of the peroxisome proliferator-activated receptor γ (PPARγ) and its coactivators steroid receptor coactivator-1 and PPAR-binding protein PBP in the brown fat, urinary bladder, colon, and breast of the mouse. Am. J. Pathol., *153:* 349–354, 1998.
18. Lefebvre, A-M., Chen, I., Desreumaux, P., Najib, J., Fruchart, J-C., Geboes, K., Briggs, M., Heyman, R., and Auwerx, J. Activation of the peroxisome proliferator-activated receptor γ promotes the development of colon tumors in C57BL/6J-APC^{Min}/+ mice. Nat. Med., *4:* 1053–1057, 1998.
19. Saez, E., Tontonoz, P., Nelson, M. C., Alvarez, J. G. A., Ming, U. T., Baird, S. M., Thomazy, V. A., and Evans, R. M. Activators of the nuclear receptor PPARγ enhance colon polyp formation. Nat. Med., *4:* 1058–1061, 1998.
20. Sarraf, P., Mueller, E., Jones, D., King, F. J., DeAngelo, D. J., Partridge, J. B., Holden, S. A., Chen, L. B., Singer, S., Fletcher, C., and Spiegelman, B. M. Differentiation and reversal of malignant changes in colon cancer through PPARγ. Med., *4:* 1046–1052, 1998.
21. Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M. Kliewer, S. A. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). J. Biol. Chem., *270:* 12953–12956, 19
22. Suh, N., Wang, Y., Williams, C. R., Risingsong, R., Gilmer, T., Willson, T. M. Sporn, M. B. A new ligand for the peroxisome proliferator-activated receptor-γ (PPARγ), GW7845, inhibits rat mammary carcinogenesis. Cancer Res., *59:* 5671–5673,
23. DuBois, R. N., Gupta, R., Brockman, J., Reddy, B. S., Krakow, S. L., and M. A. The nuclear eicosanoid receptor, PPARγ, is aberrantly expressed in c cancers. Carcinogenesis (Lond.), *19:* 49–53, 1998.
24. Devchand, P. R., Keller, H., Peters, J. M., Vazquez, M., Gonzalez, F. J., and W. The PPARα-leukotriene B_4 pathway to inflammatory control. Nature (L *384:* 39–43, 1996.
25. Jiang, C., Ting, A. T., and Seed, B. PPAR-γ agonists inhibit production of mo inflammatory cytokines. Nature (Lond.), *391:* 82–86, 1998.
26. Maggi, L. B. J., Sadeghi, H., Weigand, C., Scarim, A. L., Heitmeier, M. R Corbett, J. A. Anti-inflammatory actions of 15-deoxy-Δ12,14-prostaglandin J troglitazone. Diabetes, *49:* 346–355, 2000.
27. Su, C. G., Wen, X., Bailey, S. T., Jiang, W., Rangwala, S. M., Keilbaugh, Flanigan, A., Murthy, S., Lazar, M. A., and Wu, G. D. A novel therapy for utilizing PPAR-γ ligands to inhibit the epithelial inflammation response. J. Investig., *104:* 383–389, 1999.
28. Lehmann, J. M., Lenhard, J. M., Oliver, B. B., Ringold, G. M., and Kliewer, Peroxisome proliferator-activated receptors α and γ are activated by indomethaci other non-steroidal anti-inflammatory drugs. J. Biol. Chem., *272:* 3406–3410, 19
29. Wong, W. M., Poulsom, R., and Wright, N. A. Trefoil peptides. Gut, *44:* 890–895,
30. Mori, H., Sugie, S., Yoshimi, N., Hara, A., and Tanaka, T. Control of cell pr ation in cancer prevention. Mutat. Res., *428:* 291–298, 1999.
31. Okayasu, I., Ohkusa, T., Kajiura, K., Kanno, J., and Sakamoto, S. Promoti colorectal neoplasia in experimental murine ulcerative colitis. Gut, *39:* 87–92,
32. Cook, G. A., Yeomans, N. D., and Giraud, A. S. Temporal expression of t peptides in the TGF-α knockout mouse after gastric ulceration. Am. J. Physiol.. G1540–G1549, 1997.
33. Bradford, M. M. A rapid and sensitive method for the quantitation of micr quantities of protein utilizing the principle of protein-dye binding. Anal. Bioc *72:* 248–254, 1976.
34. Tanaka, T., Makita, H., Kawamori, T., Kawabata, K., Mori, H., Murakami, A., S K., Hara, A., Ohigashi, H., and Koshimizu, K. A xanthine oxidase inhibitc acetoxychavicol acetate inhibits azoxymethane-induced colonic aberrant crypt f rats. Carcinogenesis (Lond.), *18:* 1113–1118, 1997.
35. Tran, C. P., Cook, G. A., Yeomans, N. D., Thim, L., and Giraud, A. S. Trefoil pe TFF2 (spasmolytic polypeptide) potently accelerates healing and reduces inflat tion in a rat model of colitis. Gut, *44:* 636–642, 1999.
36. Wakabayashi, K. NSAIDs as cancer preventive agents. Asian Pac. J. Cancer Pre 97–113, 2000.
37. Tanaka, T., Kojima, T., Yoshimi, N., Sugie, S., and Mori, H. Inhibitory effect c non-steroidal anti-inflammatory drug, indomethacin on the naturally occurring cinogen, 1-hydroxyanthraquinone in male ACI/N rats. Carcinogenesis (Lond.) 1949–1952, 1991.
38. Colville-Nash, P. R., Qureshi, S. S., Willis, D., and Willoughby, D. A. Inhibiti inducible nitric oxide synthase by peroxisome proliferator-activated receptor agoı correlation with induction of heme oxygenase 1. J. Immunol., *161:* 978–984, 1
39. Wachtershauser, A., Loitsch, S. M., and Stein, J. PPAR-γ is selectively upregu in Caco-2 cells by butyrate. Biochem. Biophys. Res. Commun., *272:* 380–385, 2
40. Sarraf, P., Mueller, E., Smith, W. M., Wright, H. M., Kum, J. B., Aaltonen, L. A la Chapelle, A., Spiegelman, B. M., and Eng, C. Loss-of-function mutatior PPARγ associated with human colon cancer. Mol. Cell, *3:* 799–804, 1999.
41. Kitamura, S., Miyazaki, Y., Shinomura, Y., Kondo, S., Kanayama, S., and Matsuzaw Peroxisome proliferator-activated receptor γ induces growth arrest and differenti markers of human colon cancer cells. Jpn. J. Cancer Res., *90:* 75–80, 1999.
42. Stier, H., Fahimi, H. D., Van Veldhoven, P. P., Mannaerts, G. P., Volkl, A., Baumgart, E. Maturation of peroxisomes in differentiating human hepatoblast cells (HepG2): possible involvement of the peroxisome proliferator-activated rece α (PPAR α). Differentiation, *64:* 55–66, 1998.
43. Mehta, R. G., Williamson, E., Patel, M. K., and Koeffler, H. P. A ligan peroxisome proliferator-activated receptor γ, retinoids, and prevention of preneo tic mammary lesions. J. Natl. Cancer Inst., *92:* 418–423, 2000.
44. Watkins, P. B., and Whitcomb, R. W. Hepatic dysfunction associated with trog zone. N. Engl. J. Med., *338:* 916–917, 1998.
45. Shureiqi, I., Wojno, K. J., Poore, J. A., Reddy, R. G., Moussalli, M. J., Spindler, S Greenson, J. K., Normolle, D., Hasan, A. A. K., Lawrence, T. S., and Brenner, D Decreased 13-*S*-hydroxyoctadecadienoic acid levels and 15-lipoxygenase-1 exp sion in human colon cancers. Carcinogenesis (Lond.), *20:* 1985–1995, 1999.
46. Galloway, S. M., Johnson, T. E., Armstrong, M. J., and Ashby, J. The genetic toxi of the peroxisome proliferator class of rodent hepatocarcinogen. Mutat. Res., 4 153–158, 2000.
47. Chevalier, S., and Roberts, R. A. Perturbation of rodent hepatocyte growth contro nongenotoxic hepatocarcinogens: mechanisms and lack of relevance for hu health. Oncol. Rep., *5:* 1319–1327, 1998.
48. Xin, X., Yang, S., Kowalski, J., and Gerritsen, M. E. Peroxisome prolifera activated receptor γ ligands are potent inhibitors of angiogenesis *in vitro* and *in v* J. Biol. Chem., *274:* 9116–9121, 1999.

Hypoxia Activates a Platelet-derived Growth Factor Receptor/Phosphatidylinositol 3-Kinase/Akt Pathway That Results in Glycogen Synthase Kinase-3 Inactivation[1]

Eunice Y. Chen, Nathalie M. Mazure, Jonathan A. Cooper, and Amato J. Giaccia[2]

Department of Radiation Oncology, Center for Clinical Science Research-South, Stanford, California 94305-5152 [E. Y. C., A. J. G.]; Centre National de la Recherche Scientifique-CEREMOD, Meudon 92190, France [N. M. M.]; and Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109 [J. A. C.]

Abstract

Hypoxia initiates numerous intracellular signaling pathways important in regulating cell proliferation, differentiation, and death. In this study, we investigated the pathway that hypoxia uses to activate Akt and inactivate glycogen synthase kinase-3 (*GSK-3*), two proteins the functions of which are important in cell survival and energy metabolism. Severe hypoxia (0.01% oxygen) initiated a signaling cascade by inducing the tyrosine phosphorylation of the platelet-derived growth factor (PDGF) receptor within 1 h of treatment and increasing receptor association with the p85 subunit of phosphatidylinositol 3-kinase (PI 3-K). Hypoxia-induced signaling also resulted in PI 3-K-dependent phosphorylation of Akt on Ser-473, a modification of Akt that is important for its activation. This activation of Akt by hypoxia was substantially diminished in cells that possessed mutations in their PDGF receptor-PI 3-K interaction domain. In addition, Akt activation by hypoxia was resistant to treatment with the growth factor receptor poison suramin but was sensitive to treatment with the PI 3-K inhibitor wortmannin. Activation of Akt by hypoxia resulted in the phosphorylation of GSK-3α and GSK-3β at Ser-9 and Ser-21, two well-documented Akt phosphorylation sites, respectively, that are inactivating modifications of each GSK-3 isoform. In support of the phosphorylation data, GSK-3 activity was significantly reduced under hypoxia. In conclusion, we propose that hypoxia activates a growth factor receptor/PI 3-K/Akt cascade that leads to GSK-3 inactivation, a pathway that can impact cell survival, proliferation, and metabolism.

Introduction

The cellular response to decreased oxygen is important both in normal development and tumor progression. Previous studies have indicated that these responses are attributable to the modulation of unique intracellular signal transduction pathways. Recently, the identification of a transcription factor, HIF[3]-1, has advanced the understanding of hypoxia-induced intracellular signaling pathways (1–5). Studies of HIF-1 knockout embryos indicate that HIF-1 plays a significant role in angiogenesis and tumor growth and is also necessary for proper embryonic development, presumably through the transcriptional activation of genes involved in angiogenesis, glycolysis, and tissue remodeling (6–9). Because regulation of HIF-1 appears to be in large part attributable to posttranslational modifications of the HIF-1α subunit, which results in its stabilization, the list of proteins (iron chelators, kinases, heat shock protein 90, antioxidants, and ubiquitin) that may bind or somehow modify HIF-1 specifically under a hypoxic microenvironment is rapidly expanding (10–15). This knowledge may provide potential targets for intervention in treatment of cancers, strokes, coronary artery disease, and peripheral vascular disease.

In previous studies, we described a hypoxia-induced PI 3-K/Akt/HIF-1 pathway, leading to the activation of vascular endothelial growth factor (16). These studies suggested that Akt or protein kinase B was one of the protein kinases that lay downstream of the phospholipid products of PI 3-K and transduced the signal induced by hypoxia that results in HIF-1α stabilization in some cell types. Recently, it has been shown that 3′-phosphorylated phosphoinositides target Akt to the plasma membrane, an event that leads to the phosphorylation of Akt at two residues, Thr-308 and Ser-473, by phosphatidylinositol (3, 4, 5)P_3-dependent protein kinases (17–20). Phosphorylation of these critical residues in turn releases Akt from an inhibited conformation, thereby activating its kinase function. Some of the downstream targets of Akt are important in regulating metabolic functions such as glycogen synthesis, glucose uptake, and glycolysis. These targets include GSK-3 (21, 22), glucose transporter 4 (23, 24), and 6-phosphofructose 2-kinase (25). Other substrates of Akt are involved with its antiapoptotic function and include CED-3, c-Myc, Fas, nuclear factor-κB, and more recently, p53 (26–30).

Although a variety of signaling molecules in addition to PI 3-K (Src, Ras, and mitogen-activated protein kinase) have been shown to be modulated by hypoxia and reported to be involved with HIF-1 activation, (11, 16, 31, 32), how these molecules are initially activated by hypoxia is currently unknown. Because many of the signaling molecules activated by hypoxia are also activated by growth factors, we hypothesize that hypoxia may induce the activation of growth factor receptors and their associated pathways. The general scheme of growth factor receptor activation proceeds through a series of well-documented steps. The growth factor ligand binds to the extracellular domain of a heterodimeric receptor. The binding of the ligand results in dimerization of the receptor and autophosphorylation of the receptor at specific residues in its intracytoplasmic domain. Several Src homology 2-containing signaling molecules bind to these phosphorylated tyrosines and activate diverse downstream signal transduction pathways, the most studied of which are the mitogen-activated protein kinase and the PI 3-K pathways.

In contrast to ligand-stimulated growth factor receptor activation, UV irradiation, osmotic stress, and heat shock have been reported previously to activate growth factor receptors through ligand-independent mechanisms (33–35). Rosette and Karin (33) showed that UV light and osmotic stress caused aggregation of growth factor receptors that resulted in the activation of the JNK signaling pathway. Their study tested the hypothesis that physical stress could cause changes in the cell membrane, resulting in clustering and activation of growth factor receptors and downstream signaling cascades. Huang *et al.* (36) showed that UV treatment caused an accumulation of ROS which in turn activated several growth factor receptors, most notably the EGFR. Recent publications have proposed that hypoxia induces a mitochondria-dependent accumulation of ROS, suggesting the neces-

Received 11/21/00; accepted 1/26/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by USPHS Grants CA09302 and CA73832, awarded by the National Cancer Institute, Department of Health and Human Services.

[2] To whom requests for reprints should be addressed, at Division of Radiation and Cancer Biology, Center for Clinical Science Research-South, 269 Campus Drive, Stanford, CA 94305-5152. Phone: (650) 723-7366; Fax: (650) 723-7382; E-mail: giaccia@stanford.edu.

[3] The abbreviations used are: HIF, hypoxia-inducible factor; PI 3-K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; GSK, glycogen synthase kinase; PDGFR, platelet-derived growth factor receptor.

DEPARTMENT OF DEFENSE
BREAST CANCER RESEARCH PROGRAM

Fiscal Year 2001 Program Announcements
***** Research Funding Available *****

A $175M congressional appropriation for breast cancer research.
Join us in the fight against breast cancer.

Proposals are sought across a wide variety of disciplines including: basic, clinical, psychosocial, behavioral, sociocultural, nursing, and environmental sciences; occupational health; alternative therapies; public health and policy; and economics. Research proposals that address the needs of minority, elderly, low-income, rural, and other underrepresented and/or medically underserved populations are encouraged. Proposals also are encouraged from investigators working at Historically Black Colleges and Universities/Minority Institutions (HBCU/MI).

PROGRAM ANNOUNCEMENT I

New this year:

✦ *Breast Cancer Center of Excellence Awards*
These awards are intended to support the establishment of multi-institutional collaborations among highly accomplished scientists from diverse backgrounds to focus on a major scientific problem in breast cancer.
Proposal receipt deadline is **June 27, 2001.**

PROGRAM ANNOUNCEMENT II

Funds are available for Research, Training/Recruitment, and Infrastructure Awards

The Breast Cancer Research Program will continue to offer the following: Idea Awards, Clinical Bridge Awards, Predoctoral and Postdoctoral Traineeship Awards, Career Development Awards, Undergraduate Summer Training Program Awards, and HBCU/MI Partnership Training Awards.

New this year:

✦ An award will be offered to attract outstanding individuals from diverse fields to explore new avenues in breast cancer research.
Proposal receipt deadline is **June 13, 2001.**

Program Announcements can be downloaded from the Congressionally Directed Medical Research Programs web site, http://cdmrp.army.mil. For questions or assistance, please call 301-619-7079.